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Characterization of molecular changes in endometrium associated with chronic use of progesterone receptor modulators: ulipristal acetate vs. mifepristone

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Abstract

Ulipristal acetate (UPA) is a selective progesterone receptor modulator (PRM), which is used as an emergency contraceptive in women. Recent studies demonstrated the efficacy of a UPA contraceptive vaginal ring (UPA-CVR) as a blocker of ovulation. However the endometrium of women exposed to UPA over a six-month period display glandular changes, termed PRM-associated endometrial changes (PAECs). We, therefore, investigated whether UPA-induced PAECs are associated with altered expression of the transcription factor *HAND2* whose down regulation is observed in endometrial epithelial hyperplasia and cancer. Our results showed that while exposure to mifepristone, a well-known PRM, leads to suppression of endometrial *HAND2* expression, long-term exposure to UPA-CVR did not cause down regulation of this marker. Further studies, using human primary endometrial stromal cells, confirmed that whereas mifepristone-mediated suppression of *HAND2* elevated the levels of its downstream target fibroblast growth factor 18, UPA did not significantly alter the expression of this growth factor. A rationale for the differential regulation of *HAND2* by these PRMs was provided by our observation that mifepristone-bound progesterone receptors turn over at a faster rate than those bound to UPA. Collectively, these results support the selective effects of different PRMs and indicate that chronic exposure to UPA does not alter the *HAND2* pathway whose dysregulation is linked to complex atypical endometrial hyperplasia and cancer. The results from this study involving a limited number of clinical samples should pave the way for a larger study to determine the safety of UPA for long-term use.

Key words: Ulipristal acetate; Mifepristone; Contraception; Endometrium; *HAND2*

Introduction

It is estimated that 225 million women worldwide lack access to effective and acceptable contraceptive methods. Therefore, the development of novel clinically safe and effective methods of fertility control remains a necessity. The steroid hormone progesterone (P) acting through its nuclear receptor critically controls the ovulatory process as well as endometrial function in the human. Progesterone receptor modulators (PRM) are synthetic compounds that interact with the progesterone receptor (PR) to suppress ovulation and/or induce endometrial atrophy, resulting in amenorrhea, a condition that is perceived favorably in many cultures around the world ¹. Therefore, the development and use of PRMs as contraceptives is of particular interest.

Ulipristal acetate (UPA), also referred to as VA/CDB-2914, is a new and promising PRM ²⁻⁶. UPA has been approved as an emergency contraceptive ^{7, 8} in the United States and abroad and as a treatment for heavy menstrual bleeding due to uterine fibroids ^{9, 10} in Canada and Europe. Successful use of this PRM as an emergency contraceptive has raised the possibility that a simplified continuous delivery of UPA could improve long-term contraceptive safety and efficacy and compliance. With this goal in mind, a UPA contraceptive vaginal ring (UPA-CVR) was designed for long-term contraceptive use by the Population Council, New York. In a study conducted by the Council, healthy women with normal baseline ovulation were randomized to receive UPA-CVR for two consecutive 12-week treatment periods, followed by a recovery cycle ¹¹. The results from these studies indicated that the UPA-CVR has the potential to become an effective long-acting, user-controlled contraceptive. However, endometrial biopsies taken at the end of the treatment period displayed histological glandular changes, described as PRM-associated endometrial changes (PAECs) ¹¹. While these endometrial changes are considered to be benign due to the lack of cytological atypia ¹² an in-depth study is needed to confirm the absence of any endometrial abnormality, including hyperplasia, following chronic PRM use.

The endometrium, the innermost layer of the uterus, undergoes proliferation and differentiation in a cyclical manner in response to the steroid hormones, 17 β -estradiol (E) and P acting via their cognate receptors ¹³⁻¹⁵. While E acting via ER α functions as a mitogen and promotes the growth and proliferation of the endometrial epithelium in a cyclical fashion during the reproductive

cycle, P acting via PR inhibits E-induced epithelial proliferation and causes differentiation. Uncontrolled proliferation of the endometrial epithelium results in alterations of glandular architecture (shape and size) and an increase in endometrial gland-to-stroma ratio, leading to endometrial hyperplasia¹⁵⁻¹⁷. The majority of cases of endometrial hyperplasia are associated with compromised P signaling that fails to oppose E signaling¹⁷⁻¹⁹.

We have previously shown that the transcription factor HAND2 (Heart- and neural crest derivatives-expressed protein 2), which is regulated by the PR present in the endometrial stroma, is a key mediator of the well-known anti-proliferative effect of P on the endometrial epithelium²⁰. HAND2 suppresses the production of several stromal fibroblast growth factors (FGFs), which act in a paracrine manner via the FGF receptors to promote epithelial proliferation. Therefore, in the absence of HAND2, the endometrial epithelium undergoes unbridled FGF-induced proliferation that leads to complex atypical hyperplasia. It is of interest to note that the *HAND2* gene locus is prone to epigenetic alterations. Our recent studies revealed that the *HAND2* gene is a hypermethylated and silenced in endometrial hyperplasia and cancer²¹. When compared to other frequent DNA-based alterations in endometrial cancers, such as *p53*, *PTEN*, and *PIK3CA* mutations, *HAND2* hypermethylation was found to be the most common²¹. Since the down regulation of HAND2 expression is linked to endometrial hyperplasia and cancer, we examined the expression of this factor in endometrial biopsies of women exposed to UPA-CVR for 24 weeks. We also compared the endometrial effects of UPA with those of mifepristone, a well-known PRM.

Materials and Methods

Endometrial biopsies

Endometrial biopsy samples were obtained using either a Pipelle (Cooper Surgical, Trumbull, CT, USA) (DR, Chile) or an Explora (Cooper Surgical) (Oregon) device. A portion of each sample was placed for use in 10% neutral buffered formaldehyde for histology and immunohistochemistry studies.

***In vitro* decidualization of human endometrial stromal cells (HESC)**

Our studies involving primary HESC cultures follow the regulations stated for the protection of human subjects participating in clinical research and are approved by the institutional review boards of Emory University, Wake Forest University (Winston-Salem, North Carolina), and the University of Illinois at Urbana-Champaign (UIUC). Endometrial samples from the early proliferative stage of the menstrual cycle were obtained by Pipelle biopsy at Emory University and Wake Forest Medical Centers from fertile, regularly cycling volunteers with no sign of uterine abnormality, providing written informed consent as described previously^{21, 22}.

Cells were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 5% (vol/vol) fetal bovine serum (Hyclone), 50 µg/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). For *in vitro* differentiation, the cells were treated with differentiation cocktail composed of 10 nM E (Sigma), 1 µM progesterone (Sigma), 0.5 mM 8-bromoadenosine-cAMP (Sigma), 10 µM UPA or mifepristone in DMEM/F-12 medium (Invitrogen) supplemented with 2% (vol/vol) charcoal dextran-stripped fetal bovine serum for 0-6 days. At the end of the culture (2 or 6 days), the cells were detached from the plates, counted, and stored at -80°C for RNA extraction. Additionally some cells were fixed for immunocytochemical (ICC) analysis. In some experiments, the cells were treated with differentiation cocktail composed of 10 nM E, 1 µM progesterone, 0.5 mM 8-bromoadenosine-cAMP, 5 µM UPA or mifepristone in DMEM/F-12 medium supplemented with 2% (vol/vol) charcoal dextran-stripped fetal bovine serum for 0-6 days. Cultures were terminated at days 2 to 6 for RNA extraction.

Chemicals, reagents, and antibodies

Progesterone (P), 17β-estradiol (E), naphthol AS-MX phosphate, Fast Blue RR (4-benzoylamino- 2,5-dimethoxyaniline diazonium), collagenase, pancreatin, dimethyl sulfoxide (DMSO), 8-bromoadenosine 3', 5'-cyclic monophosphate salt (cAMP), and Trypan blue were purchased from Sigma. Hanks Balanced Salt Solution (HBSS), dispase, Dulbecco's modified Eagle medium-F12 medium HEPES, no phenol red (DMEM/F12), Penicillin-Streptomycin, and Fungizone, were purchased from Life Technologies. Fetal bovine serum (FBS) was purchased from Fisher Scientific. Fluoromount-G with DAPI was purchased from eBiosciences.

Endometrial sections or endometrial stromal cells were incubated with one or more of the following primary antibodies: heart- and neural crest derivatives-expressed transcript 2 (HAND2, 1:250, Santa Cruz Biotechnology antibody SC-9409), FGF18 (1:100, Santa Cruz Biotechnology antibody SC-393471), PR (1:100, DAKO-A0098), and PRB²² (1:300, Cell signaling CST-31575). The fluorescent-tagged secondary antibodies and normal donkey serum were purchased from Jackson ImmunoResearch. The following secondary antibodies were used: rhodamine or Cy3 donkey anti-rabbit, 488 donkey anti-rabbit, 488 donkey anti-mouse, 488 donkey anti-goat, and Cy3 donkey anti-rat.

Immunohistochemistry (IHC) and immunocytochemistry (ICC)

Paraffin-embedded endometrial biopsy sections were subjected to IHC as described previously. Tissue sections were deparaffinized in xylene, rehydrated through a graded series of ethanol, and washed in tap water. For most of the immunostaining, antigen retrieval was performed in a pressure cooker in 10 mM sodium citrate buffer (pH 6.0) for 20 min and then the slides were cooled to room temperature. The sections were washed between steps (three times for 5 min each) using 1x phosphate-buffered saline solution containing 0.05% Tween 20 (PBS-T). Nonspecific binding was inhibited by incubating the sections with 10% normal serum for 1 h at room temperature. After the serum block, sections were incubated overnight at 4°C with the diluted antibody solution in PBS-T containing 1% normal serum.

Labeling was visualized by incubation with a fluorescent-tagged secondary antibody for 1 h at room temperature. All incubations were done using a humidified chamber protected from light. Slides were mounted using a mounting solution containing DAPI. Pictures were taken using the Olympus BX51 microscope equipped for fluorescent imaging and connected to a Jenoptik ProgRes C14 digital camera with c-mount interface containing a 1.4 Megapixel CCD sensor. Fluorescent images were processed and merged using Adobe Photoshop Extended CS6 (Adobe Systems). HSCOREs were determined as described previously²³.

For ICC analysis of HESC, cells were fixed in 10% NBF for 10 min, and then washed with PBS. Cells were then permeabilized using PBS containing 0.1% Triton X for 10 min at room temperature. Nonspecific binding was inhibited by incubating the sections with 10% normal

serum for 1 h at room temperature. After the serum block, the cells were incubated overnight at 4°C with the diluted antibody solution in PBS containing 1% normal serum. Labeling was visualized by incubation with a fluorescent-tagged secondary antibody for 1 h at room temperature. One drop of mounting solution containing DAPI was added to each well to stain the nucleus. Pictures were taken using the Olympus Ix70 inverted microscope adapted to a Diagnostic Instrument digital camera containing a 2.0 Megapixel CCD sensor. Fluorescent images were merged and processed using Adobe Photoshop Extended CS6.

Quantitative real time PCR analysis (qPCR)

Total RNA was isolated from endometrial cells using a standard TRIzol-based protocol. The RNA concentration of each sample was determined at 260 nm using a Nanodrop ND1000 UV-Vis spectrophotometer (Nanodrop Technologies). RNA samples were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Real time quantitative PCR (qPCR) reactions were carried out using SYBR-green master mix (Applied Biosystems) in a 7500 Applied Biosystems Real-time PCR machine (Applied Biosystems). For each sample, the mean threshold cycle (Ct) was calculated from Ct values obtained from three replicates. The normalized ΔCt in each sample was calculated as mean Ct of target gene subtracted by the mean Ct of the reference gene. The fold change of gene expression in each sample relative to a control was generated using the $2^{-\Delta\Delta Ct}$ mathematical model for relative quantification of quantitative PCR. The mean fold induction and SEM were calculated from at least three or more independent experiments. The housekeeping gene RPLP0 (36B4), which encodes a ribosomal protein, was used as a reference gene.

Statistical analyses

Experimental data for studies related to UPA-CVR were collected from 12 independent subjects. For each subject, 4 endometrial biopsy samples were obtained. Biopsy 1 was an endometrial specimen obtained before administration of UPA-CVR, biopsies 2 and 3 were endometrial specimens obtained after each 12-week period in which UPA-CVR released UPA daily, and biopsy 4 was obtained following a 4-week post-treatment recovery period. Results from mifepristone studies were obtained from 6 independent clinical samples. Data related to primary HESCs were collected from 3 independent clinical samples, which were subjected to the same

experimental conditions. All numerical data are expressed as mean \pm SEM. When experimental samples were compared with control samples, statistical significance between the control and experimental sample was determined using the Student *t* test. A *P* value of $\leq .05$ was considered to be significant.

Results

Expression of HAND2 is unaltered in human endometrial biopsies exposed to UPA-CVR

Human endometrial biopsies were obtained from three different clinics located in the United States, Dominican Republic, and Chile. We have analyzed a total of 12 independent subjects. For each subject, 4 endometrial biopsy samples (biopsies 1-4) were obtained. Biopsy 1 is an endometrial specimen obtained before administration of UPA-CVR during the luteal phase based on urine LH determinations. Biopsies 2 and 3 are endometrial specimens obtained after each 12-week period in which UPA-CVR released 1.5 mg or 2.5mg UPA daily. Biopsy 4 was obtained following a 4-week post-treatment recovery period in the luteal phase, determined as above. Figure 1 shows representative endometrial samples at baseline, before administration of UPA-CVR (panel A), after exposure to UPA-CVR (panel B), and in the recovery phase (panel C). Baseline samples show normal mid-secretory phase endometrium. Upon exposure to UPA-CVR, the glands show variable cystic dilatation, mildly disordered architecture, non-physiological secretory appearances, and coexistent mitoses and apoptotic bodies. The stroma is compact, non-decidualized and contains occasional thick-walled vessels. These features are characteristic of PRM-associated endometrial changes or PAECs. In the recovery phase, the endometrium exhibits normal early secretory phase appearances.

To examine the molecular changes in the endometrium following prolonged exposure to UPA-CVR, we investigated the expression of HAND2 in the biopsy specimens. An intense nuclear staining specific to HAND2 was observed in the endometrial stromal cells of pre-treatment biopsy-1 specimen (Figure 2). This expression of stromal HAND2 remained unaltered in the biopsies exposed to UPA (biopsy-2, and -3, Figs. 2B and C) and in the post-treatment biopsy

specimen (biopsy-4, Fig. 2D). To more accurately quantify the immunohistochemical findings, HSCOREs were analyzed. HSCOREs of endometrial HAND2 immunostaining revealed no significant changes across the treatment period (Figure 3). These results indicate that a CVR releasing 1.5 or 2.5mg/day of UPA for 24 weeks does not affect HAND2 expression.

Expression of HAND2 is reduced in human endometrial biopsies exposed to mifepristone

We also analyzed the expression of HAND2 in endometrial biopsies collected from women exposed to mifepristone, a well-known PRM. In this study, 50 mg of oral mifepristone was administered every other day for 12 weeks. Endometrial biopsies were taken in the secretory phase of the last week of mifepristone treatment. Luteal phase biopsies from unexposed women demonstrated robust expression of HAND2 in the nuclei of stromal cells, as expected. In contrast, endometrial biopsies of women treated with mifepristone showed a significant decline in the expression of HAND2 (Figure 4). Quantification of HAND2 immuno-positive cells in the stroma revealed greater than 80% reduction in HAND2 expression in mifepristone-exposed biopsies when compared to unexposed controls. Collectively, these results suggest that UPA-CVR and mifepristone have differential effects on endometrial HAND2 expression. It is possible that the differences are due to the pharmacology of the PRM compounds, their doses, duration or route of administration.

UPA and mifepristone differentially regulate *HAND2* and *FGF18* expression in cultured human endometrial stromal cells

To directly examine the pharmacological effects of UPA and mifepristone on HAND2 expression in the endometrial stroma under identical study conditions, we utilized a well-established human endometrial stromal cell culture system. In this system, undifferentiated stromal cells isolated from human endometrial biopsies (HESC) obtained from normal women in the proliferative stage of the menstrual cycle were placed in culture and subjected to decidualization in response to a hormonal mixture containing 10 nM E, 1 μ M P, and 0.5 mM 8-bromo-cAMP^{24,25}. Under the treatment conditions, cells were treated with the hormonal mixture with or without 10 μ M UPA or mifepristone. HESCs were cultured in the presence of hormones

with or without PRMs for up to six days. *HAND2* mRNA expression was reduced when endometrial stromal cells were exposed to UPA or mifepristone for two days, compared to cells not treated with PRMs (Figure 5). While UPA exposure reduced *HAND2* expression by 20%, treatment with mifepristone resulted in almost 40% reduction in *HAND2* expression ($P<0.05$). Further, the inhibitory effect of mifepristone on *HAND2* expression increased in severity with longer duration of treatment. Stromal cells exposed to mifepristone for six days displayed more than 80% reduction in *HAND2* expression when compared to untreated control cells. In contrast, treatment with UPA for six days had milder effects, resulting in a 30% reduction in *HAND2* expression ($P<0.05$). Consistent with the RNA profile, immunocytochemical (ICC) analysis revealed similar reductions in *HAND2* in the UPA (Figure 6, panel B)- and mifepristone (Figure 6, panel C) -exposed endometrial stromal cells compared to UPA- or vehicle-treated stromal cells (Figure 6, panel A).

To further investigate the differential effects of UPA and mifepristone on *HAND2* expression, we reduced the levels of PRMs from 10-fold to 5-fold molar excess of P. Human endometrial stromal cells were cultured in the presence of hormones with or without 5 μ M UPA or mifepristone for up to six days. *HAND2* mRNA expression was monitored on day 2, day 3, day 4, day 5, and day 6 after initiation of the culture. As shown in Figure 7, treatment of HESC with 5 μ M UPA did not affect the expression of *HAND2* on days 2 to 6 upon initiation of the culture. By contrast, administration of 5 μ M mifepristone led to a significant down regulation of *HAND2* expression in HESCs. The decline in *HAND2* expression was evident on day 2 and continued up to day 6 of culture.

Our previous studies have shown that *Hand2* expression in the stroma suppresses the production of fibroblast growth factors (FGFs) and inhibits cell proliferation²⁰. In the absence of *Hand2*, continued induction of FGFs in the stroma activates FGF receptor (FGFR) signaling in the epithelium to promote cell proliferation²⁰. Consistent with this observation, a recent study reported a decrease in *HAND2* expression and marked increase in the levels of FGF18 in human endometrial adenocarcinoma²⁶. Mifepristone-treated endometrial stromal cells demonstrated a marked increase in the levels of FGF18 mRNA (Figure 8, upper panel) and protein (Figure 8, lower panel) compared to vehicle-treated controls. In contrast, endometrial stromal cells exposed

to UPA did not exhibit alterations in FGF18 expression (Figure 8). Collectively, these results support our *in vivo* findings and indicate that endometrial stromal cells cultured with mifepristone or UPA under identical *in vitro* conditions exhibit differential effects on HAND2 and FGF18 expression.

Mifepristone and UPA differentially affect PR stability in human endometrial stromal cells

Both UPA and mifepristone are known to regulate the function of a tissue by modulating the activity of PR, so it is interesting that endometrial stromal cells display differential gene expression when exposed to the same concentrations of these two PRMs. We considered the possibility that the stability of endometrial PR might be regulated differentially by mifepristone and UPA. To investigate this possibility, we determined the expression of total PR protein in progesterone-, UPA-, or mifepristone-treated HESC by ICC. Cells exposed to progesterone or UPA for 6 days displayed prominent nuclear PR staining, while those treated with mifepristone showed markedly reduced levels of PR (Figure 9).

Our recent studies revealed that the PR isoform PR-B plays a predominant functional role during human endometrial stromal differentiation by controlling the expression of a large number of target genes, including HAND2²¹. We noted distinct expression of PR-B²² in the nuclei of progesterone- or UPA-treated stromal cells (Figure 10). In contrast, nuclei of stromal cells exposed to mifepristone were mostly devoid of PR-B expression. Taken together, these results are consistent with our view that UPA and mifepristone differentially affect PR stability in human endometrial stromal cells and this is reflected in altered expression of PR target genes, such as HAND2, in response to these ligands in the endometrial stroma.

Discussion

A critical balance of E and P drives proper endometrial stromal-epithelial crosstalk and maintains normal uterine physiology. Disruption of PR function results in unopposed E action, causing epithelial hyperplasia and potentially carcinoma¹⁷⁻¹⁹. HAND2, a PR-regulated gene in the stromal cells, mediates the antiproliferative action of P to regulate endometrial epithelial

function. Loss of the antiproliferative actions of P in the uterus has been linked to E-dependent endometrial cancer²⁷. Indeed, our recent study showed that the *HAND2* gene is hypermethylated in premalignant endometrial lesions compared to normal endometrium and its expression is suppressed in endometrial hyperplasia and cancer²¹. *HAND2* has therefore emerged as a key molecular alteration in endometrial cancer that could potentially be employed as a biomarker for early detection of endometrial cancer.

To determine the clinical utility of UPA as a long-term contraceptive, it is critical to assess whether this compound, which effectively blocks PR action and ovulation, also alters the critical balance of E and P in the endometrium. Evaluation of endometrial histology following chronic UPA treatment revealed the presence glandular changes, known as PAECs, which did not show any cytological atypia, a characteristic feature of hyperplasia and cancer. However, routine histological examination of the endometrium may not provide molecular information related to a subtle imbalance of E- and P-dependent signaling that may arise due to PRM exposure. In this study, we show that the expression of *HAND2*, which critically regulates the balance of P- and E- dependent signaling in the endometrium, is unaffected in women exposed to UPA-CVR continuously for 24 weeks. Since downregulation of endometrial *HAND2* has been linked to complex atypical hyperplasia and cancer, unaltered expression of this factor gives us confidence that exposure to the studied dose of UPA by the vaginal route of administration does not disrupt the critical balance of E- and P- dependent signaling necessary for normal endometrial physiology.

In contrast, we found that endometrial biopsies from women treated with mifepristone for 12 weeks displayed a dramatic downregulation of *HAND2*. Differential effects of UPA and mifepristone on *HAND2* expression were confirmed in endometrial stromal cells cultured under identical conditions, suggesting distinct mechanisms underlie the actions of these PRMs. Analysis of PR in endometrial stromal cells following in vitro exposure to PRMs demonstrated that mifepristone down regulates the PR levels, whereas equivalent molar concentrations of UPA did not have these effects. This suppression of cellular PR levels by mifepristone is consistent with previous reports that addition of mifepristone to a progestogen-only regimen of contraception leads to downregulation of PR-B²⁸. Additionally, recent studies have

demonstrated that administration of mifepristone to an endometrial co-culture system causes suppression of PR expression compared to vehicle-treated controls ²⁹. While the mechanism by which mifepristone causes PR turnover remains unclear, we believe that this downregulation of PR is in part responsible for the dramatic suppression of HAND2 expression observed in response to mifepristone compared to UPA.

We have previously shown that HAND2 mediates the antiproliferative effects of P by suppressing the production of the FGF growth factors that mediate the growth-inducing effects of E on the endometrial epithelium. In the E-dominant proliferative endometrium, FGFs secreted from the stroma act on the FGFR(s) in the epithelium to promote proliferation ²⁰. Following ovulation and in response to P production and signaling, HAND2 is induced in stromal cells, causing inhibition of FGF synthesis and attenuation of epithelial proliferation. Disruption of PR function in the endometrium therefore runs the risk of increasing FGF signaling, leading to inappropriate uterine epithelial growth, hyperplasia and cancer. Similar findings were noted in the epithelial glands of rhesus macaques treated with mifepristone ³⁰. Indeed, a recent study has shown downregulation of HAND2 and upregulation of FGF18 in human endometrial adenocarcinoma ²⁶. We demonstrate that administration of mifepristone to cultured endometrial stromal cells caused inhibition of HAND2 expression and a concomitant enhancement of FGF18 expression. However, treatment of endometrial stromal cells with UPA did not significantly affect the expression of either HAND2 or FGF18, further confirming that UPA does not significantly alter the P-dependent antiproliferative pathways in the endometrium.

In summary, this study shows that UPA and mifepristone exhibit differential effects on endometrial gene expression in vivo and in vitro, apparently due to differences in stability of PRs in response to these PRMs. It also confirms that chronic exposure to UPA-CVR over a 24-week period does not lead to adverse effects, such as suppression of the expression of HAND2, which is reported to occur in endometrial hyperplasia and cancer. The results from this study involving a limited number of clinical samples should pave the way for a larger study to determine the safety of UPA for long-term use.

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Figure legends

Fig. 1: Endometria of women exposed to UPA-CVR display PRM-associated endometrial changes (PAECs). Haematoxylin & eosin staining of endometrial sections obtained from normal mid-secretory phase endometrium (panel A), UPA-CVR releasing 2.5mg UPA daily for two consecutive 12-week treatment periods (panel B), and post-treatment recovery period in the luteal phase (panel C). Note cystic dilatation, mildly disordered architecture, and non-physiological secretory appearances in the endometria of women with UPA-CVR. These endometrial samples are part of the large clinical trial. Representative images are shown.

Fig. 2: Expression of HAND2 in human endometrial biopsies exposed to UPA-CVR for 24 weeks. Immunohistochemical localization of HAND2 in endometrial sections before and after exposure to UPA-CVR. A total of 12 independent subjects were analyzed and for each subject, 4 endometrial biopsy samples were obtained (N=48). Panel A represents endometrial specimen obtained during the luteal phase before administration of UPA-CVR. Panels B and C indicate endometrial specimens obtained after each 12-week period with UPA-CVR releasing 1.5mg or 2.5mg UPA daily. Panel D represents endometrial specimen collected during the luteal phase following a post-treatment recovery period. Panel E shows endometrial sections from a biopsy sample after a 12-week exposure to UPA-CVR and subjected to IHC protocol omitting the primary antibody. Red staining indicates positive staining for HAND2 in endometrial sections. Representative images are shown. S and E indicate stroma and epithelium respectively.

Fig. 3: HAND2 expression is unaltered in human endometrial biopsies exposed to UPA-CVR. The percentages of the immunostaining positive cells for HAND2 were analyzed by ImageJ software. The values represent mean \pm SEM of twelve independent samples (N=5 for UPA-CVR releasing 1.5 mg and N=7 for UPA-CVR releasing 2.5mg UPA daily) with a total of N=48 clinical samples. No obvious dose-response effects were noted between the two doses.

Fig. 4: Expression of HAND2 is reduced in human endometrial biopsies exposed to mifepristone. **Upper:** Immunohistochemical analysis of HAND2 in human endometrium before (panel A) and after administration of 50 mg of oral mifepristone every other day for 12 weeks of the menstrual cycle (panel B). Representative images are shown. **Lower:** HSCORES of HAND2-positive cells in the endometrium revealed a significant reduction in HAND2 expression in mifepristone-exposed biopsies when compared to unexposed controls (N=6). B indicates baseline (0 wks) and after 12 weeks (12 wks) indicates end of treatment, respectively, and shows a significant decrease ($P<0.02$).

Fig. 5: UPA and mifepristone differentially regulate *HAND2* mRNA expression in human endometrial stromal cells. Primary cultures of human stromal cells were grown in Dulbecco's modified Eagle's medium/F-12 medium containing 5% charcoal-stripped fetal bovine serum. The cells were treated with a hormone mixture containing 10 nM E, 1 μ M P, 0.5 mM 8-bromo-cAMP, and 10 μ M UPA, mifepristone or vehicle for 6 days. Cells were harvested 2 days (left panel) or 6 days (right panel) after addition of hormone mixture. Total RNA was isolated and subjected to qPCR using primers for *HAND2*. The level of *Rplp0* was used as an internal control to normalize gene expression. The values are presented as the mean fold induction \pm SEM, $P<0.05$.

Fig. 6: UPA and mifepristone differentially regulate *HAND2* protein expression in human endometrial stromal cells. Immunocytochemical analysis of HAND2 in stromal cells during *in vitro* decidualization. Panels represent primary cultures of human endometrial stromal cells cultured in the absence of UPA or mifepristone (A), in the presence of UPA (B), in the presence of mifepristone (C) for 6 days. Representative images from three independent experiments are shown.

Fig. 7: UPA and mifepristone differentially regulate *HAND2* mRNA expression in human endometrial stromal cells. Primary cultures of human stromal cells were treated with a hormone mixture containing 10 nM E, 1 μ M P, 0.5 mM 8-bromo-cAMP, and 5 μ M UPA, mifepristone or vehicle for 6 days. Cells were harvested 2, 3, 4, 5, and 6 days after addition of hormone mixture. Total RNA was isolated and subjected to qPCR using primers for *HAND2*. The level of *Rplp0*

was used as an internal control to normalize gene expression. The values are presented as the mean fold induction \pm SEM, * $P < 0.05$, ** $P < 0.01$.

Fig. 8: Downregulation of FGF18 expression in response to mifepristone in human endometrial stromal cells. Human endometrial stromal cells were subjected to differentiation in response to 0.5 mM 8-bromo-cAMP, 1 μ M P, 10 nM E, and 10 μ M UPA or mifepristone for 6 days. **Upper.** Total RNA was isolated and subjected to qPCR using primer for *FGF18*. Y-axis indicates fold induction. The level of *Rplp0* was used as an internal control to normalize gene expression. The data are represented as the mean fold induction \pm SEM from three separate samples. **Lower.** Immunocytochemical analysis of FGF18 expression in endometrial stromal in the absence of UPA or mifepristone (left panel), in the presence of UPA (middle panel) and in the presence of mifepristone (right panel). Representative images are shown.

Fig. 9: PR stability in response to UPA or mifepristone in human endometrial stromal cells. Primary cultures of human stromal cells were treated with a hormone mixture containing 10 nM E, 1 μ M P, 0.5 mM 8-bromo-cAMP, and 10 μ M UPA, mifepristone or vehicle for 6 days. Immunocytochemical analysis of PR in endometrial stromal in the absence of UPA or mifepristone (left panel), in the presence of UPA (middle panel) and in the presence of mifepristone (right panel) are shown. Representative images are shown.

Fig. 10: PR-B stability in response to UPA or mifepristone in human endometrial stromal cells. Primary cultures of human stromal cells were treated with a hormone mixture containing 10 nM E, 1 μ M P, 0.5 mM 8-bromo-cAMP, and 10 μ M UPA, mifepristone or vehicle for 6 days. PR-B expression in endometrial stromal in the absence of UPA or mifepristone (left panel), in the presence of UPA (middle panel) and in the presence of mifepristone (right panel) are shown. Representative images are shown.

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Fig. 1

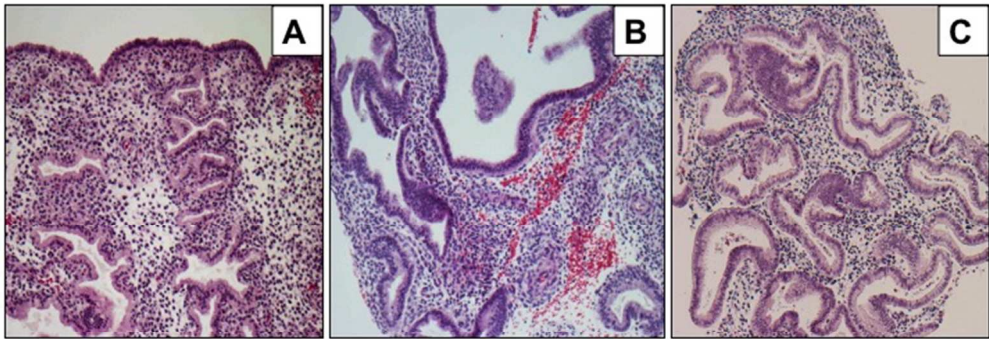
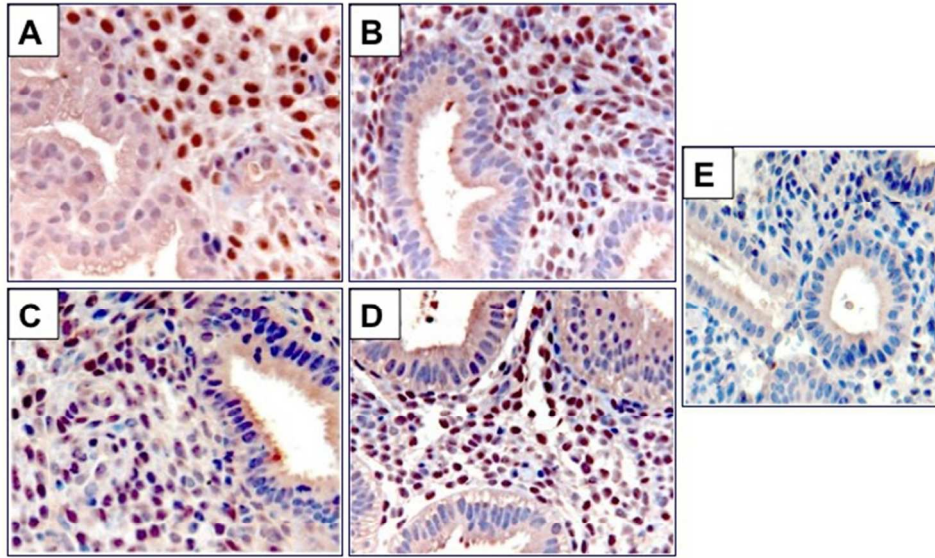


Fig.1

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Fig. 2**Fig.2**

254x190mm (72 x 72 DPI)

Fig. 3

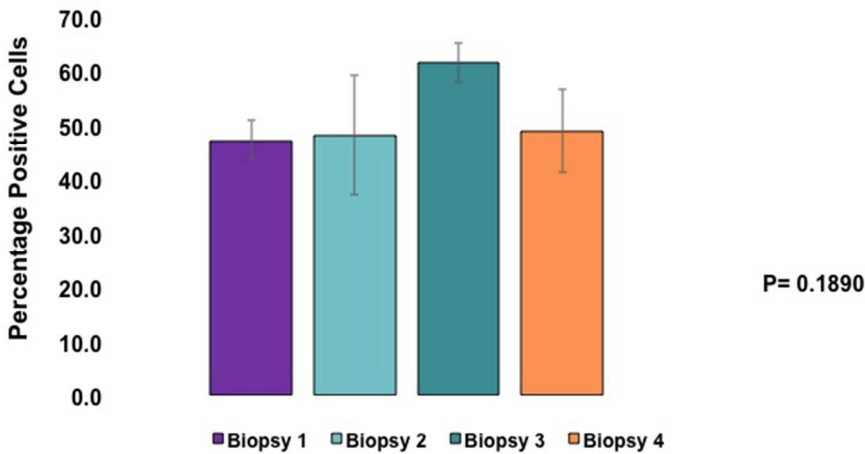
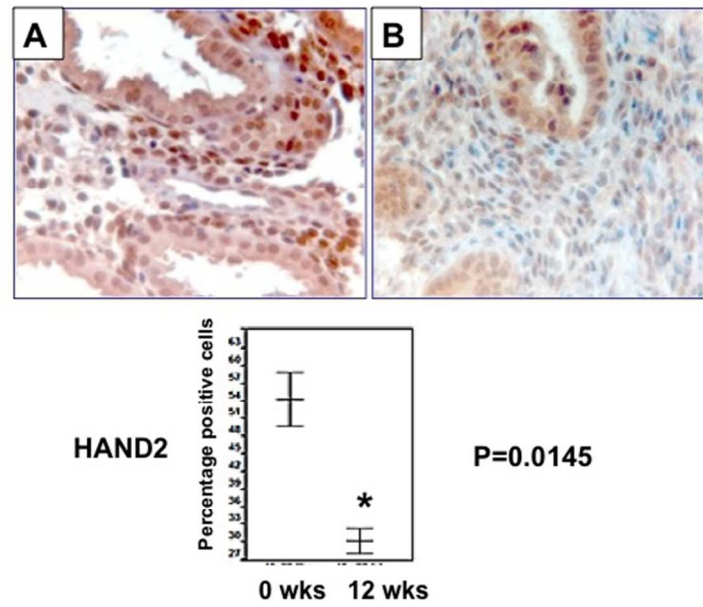


Fig.3

254x190mm (72 x 72 DPI)

Fig. 4**Fig.4**

254x190mm (72 x 72 DPI)

Fig. 5

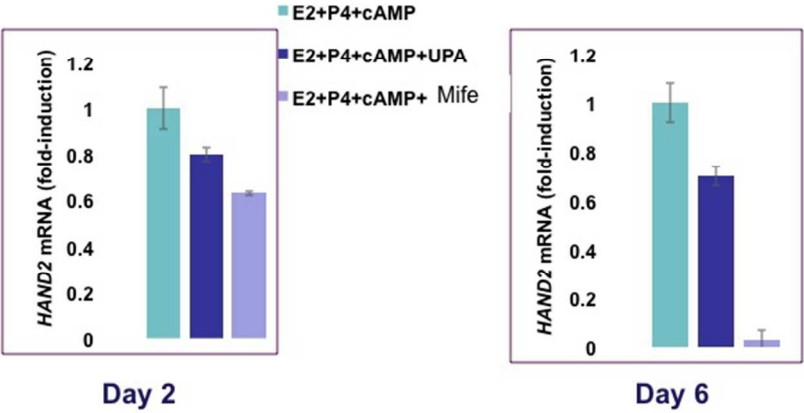
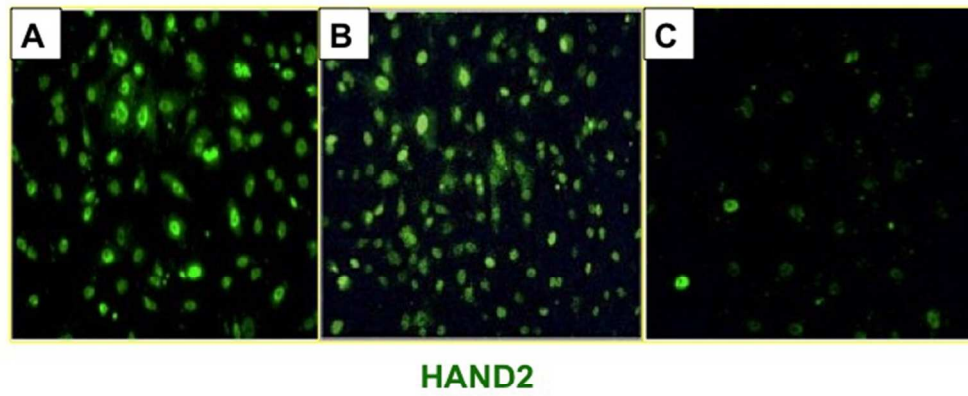


Fig.5

254x190mm (72 x 72 DPI)

Fig. 6**Fig.6**

254x190mm (72 x 72 DPI)

Fig. 7

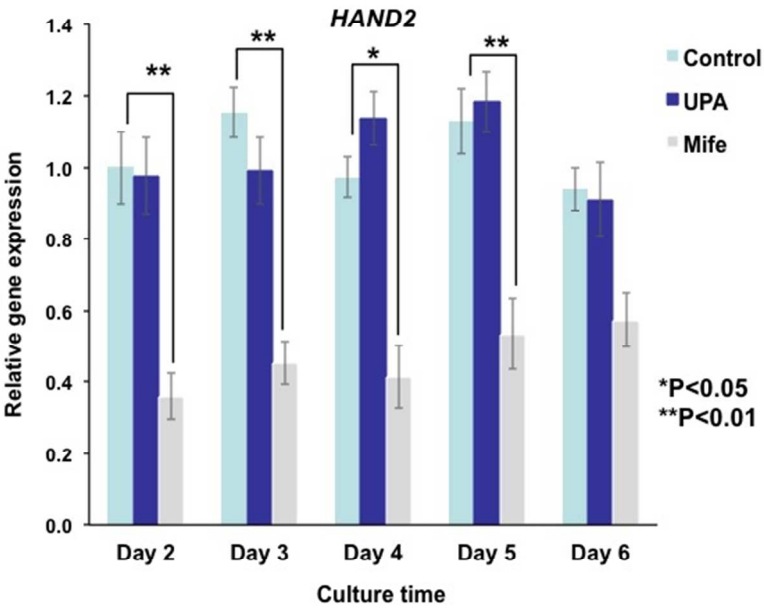
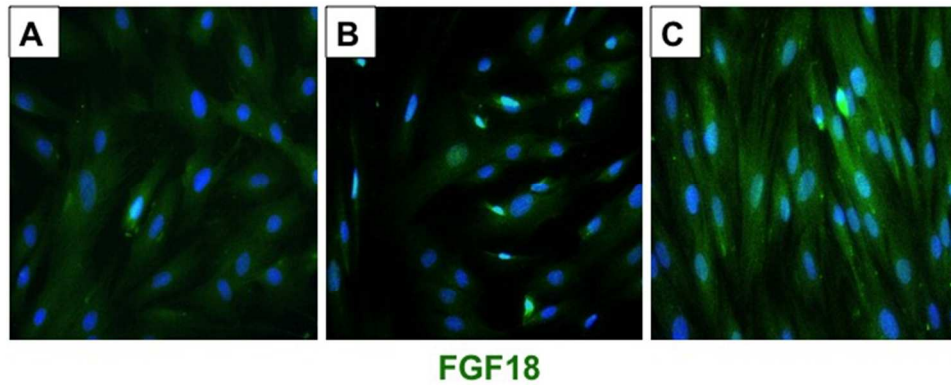
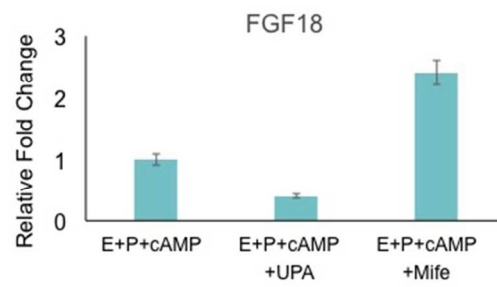


Fig.7

254x190mm (72 x 72 DPI)

Fig. 8**Fig.8**

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Fig. 9

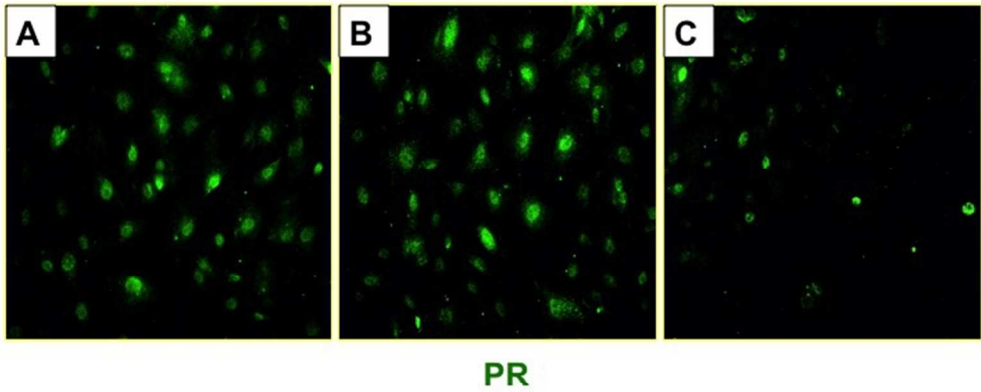


Fig.9

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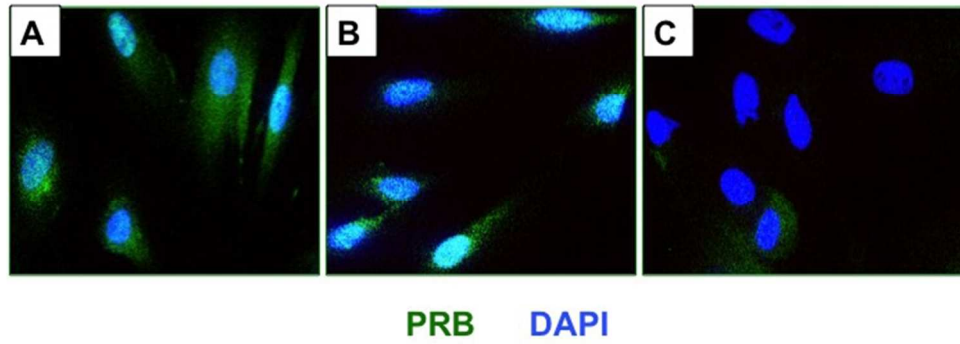
Fig. 10

Fig.10

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